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PRELIMINARY STUDIES ON THE EFFECT OF FEEDING DURING WHOLE SEDIMENT BIOASSAYS USING *Chironomus riparius* LARVAE

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ABSTRACT

Current protocols for freshwater sediment bioassays require feeding, which will likely alter the exposure to sediment-associated contaminants. To determine the potential change in exposure brought about by adding uncontaminated food, whole sediment contaminant bioaccumulation by the midge, *Chironomus riparius*, was determined in the presence and absence of added food. Lake Michigan sediment was dosed with radiolabeled polycyclic aromatic hydrocarbons (PAHs) and/or DDT and *trans*-chlordane. Three groups of organisms (Feeding Levels I and II plus a control) were exposed in static assays. After two-, four-, seven-, and ten-day exposures, individual larvae were analyzed for contaminant concentration, mass, and total lipid content. After 7 to 10 days, accumulation of pyrene and benzo(a)pyrene was significantly greater with feeding, while larvae exposed to chrysene accumulated significantly less contaminant when fed, compared to controls. No feeding-related differences in accumulation of the two insecticides were observed. Significant differences in larval mass between test animals and controls were observed only with pyrene-dosed sediment. Larval lipid content tended to remain constant throughout the exposures and did not differ between fed and unfed organisms. Thus, it appears that contaminant bioavailability can be altered by the addition of uncontaminated food. However, this phenomenon appears to be compound-specific and not broadly predictable.

INTRODUCTION

Toxicity and bioaccumulation tests are methods commonly used to determine the potential impact of sediment-associated contaminants. Various guidelines and standards for bioassays concerning the type and species of indicator organisms to be used, length of exposure intervals, and experimental design have been established (ASTM 1991 *a, b,* U. S. EPA 1989). However, there is presently a lack of consensus regarding the actual conditions for conducting sediment bioassays, such as whether to use static systems or to regularly renew overlying water, and to feed or not to feed indicator organisms during an assay. The lack of standardization has the potential to create variable and uninterpretable results among laboratories that use different protocols.

Guidelines established by the American Society for Testing and Materials (ASTM) for conducting sediment toxicity tests with marine and estuarine amphipods state that animals do not require supplementary feeding for assays of short duration, although feeding may be required for tests longer than ten days (ASTM 1991 *b*). Alternatively, ASTM standards for conducting toxicity tests with freshwater invertebrates state that feeding should be included for the duration of the tests (ASTM 1991 *a*), although standard food regimens, amounts, and frequencies of feeding have not been established. Further, proposed guidelines for determining the bioaccumulation of sediment-associated contaminants by benthic invertebrates recommend that food not be given during a test (U. S. EPA 1989). It has been suggested that adding food during bioaccumulation tests

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might alter the exposure of test organisms to sediment-associated contaminants by increasing organic carbon partitioning, due to the addition of this exogenous material. Organisms that preferentially feed on food added to exposures may disrupt normal sediment ingestion and change tissue absorption by altering the physiological partitioning of contaminants within the gut (U. S. EPA 1989, 1992). Feeding can also potentially alter elimination of contaminants, such that elimination may be enhanced when organisms feed on uncontaminated material. Thus, feeding of indicator species is hypothesized to reduce the accumulation and therefore the toxicity of contaminants. However, some studies have shown that feeding is necessary to avoid a high percentage of false positives in toxicity tests, especially when nutrient-poor substrates are used (Ankley *et al.* 1993 *a, b*, Phipps *et al.* 1993).

This study was designed to determine possible accumulation effects resulting from the addition of food during whole sediment bioassays, using a variety of organic contaminants with fourth instar larvae of the midge, *Chiromomus riparius*. *Chiromomus* larvae have been recommended for use in both sediment toxicity and bioaccumulation tests (McCahon and Pascoe 1988, U. S. EPA 1989) and have the potential to be used as indicator species in numerous bioassays involving hazard assessment. Our objective was to temporally monitor uptake of several hydrophobic organic contaminants in organisms with and without the addition of food to determine differential accumulation of contaminants in test (fed) and control (unfed) replicates. In addition, wet weight and lipid content of animals were analyzed to determine differences between animals receiving and not receiving added food during the study.

MATERIALS AND METHODS

The contaminants used in the study included ^{14}C-radiolabeled *trans*-chlordane (13.7 mCi/mmol, Velsicol Chemical Co., Memphis, TN), 4,4' [^{14}C]-DDT (11.8 mCi/mmol, Sigma Chemical Company, St. Louis, MO), [^{3}H]-benzo(*a*)pyrene (BaP, 69.0 Ci/mmol, Amersham Ltd., Amersham, UK), [^{3}H]-chrysene (340.0 mCi/mmol, Chemsyn Science Laboratories, Lenexa, KS), and [^{3}H]-pyrene (25.2 Ci/mmol, Chemsyn Science Laboratories, Lenexa, KS). All compounds were dissolved in an acetone carrier. Compound radiopurity was greater than 97% in all compounds prior to use as determined by thin layer chromatography, using either benzene:ethyl acetate (3:1, v:v, DDT and *trans*-chlordane) or hexane:benzene (8:2, v:v, BaP, chrysene, and pyrene) solvents and by liquid scintillation counting (LSC). All solvents were of HPLC grade. Analytical procedures were performed under gold fluorescent light ($\lambda \geq 500$ nm) to minimize photodegradation of the polycyclic aromatic hydrocarbons (PAHs).

Sediment was collected at a 45-m depth in Lake Michigan by Ponar grab approximately 8 km off the coast of Grand Haven, MI. The sediment was passed through a 1-mm sieve to remove debris and indigenous organisms. A sediment-water slurry was made by diluting wet sediment with Lake Michigan water in a 1:4 sediment to water ratio (w/v). Radiolabeled chemicals were added to the slurry drop by drop in a minimal amount of acetone carrier (<1 ml/L wet sediment) while being stirred on a mechanical stirrer at room temperature for four hours. Two sediments were prepared with dual-labeled compounds in the following combinations: BaP/*trans*-chlordane and DDT/chrysene. One sediment was single-labeled with pyrene. After stirring, sediment slurries were left to settle at 4°C for 48 hours. After settling, overlying water was decanted and the sediment was washed with another four volumes of lake water. The mixture was again stirred by mechanical stirrer at room temperature for another four hours, then left to settle in the dark at 4°C for one week (first DDT/chrysene assay), one month (pyrene assay), three months (second DDT/chrysene assay), and four months (*trans*-chlordane/BaP assay) before testing.

C. riparius were reared in the laboratory at ambient temperature (21 - 23°C) on a substrate of shredded brown paper towels and a diet of ground Tetramin® (TetraWerk, Germany) and Cerophyl® (AgriTech, Kansas City, MO), according to ASTM guidelines (ASTM 1991*a*). Fourth instar larvae were removed from the culture

aquaria and placed in environmental chambers with the same photo period (18D:6N) and feeding schedule as in the original culture aquaria. All exposures in this study were conducted at 10°C to slow larval development, so that adult emergence would not resume during the assays. Larvae were acclimated to 10°C by lowering the temperature by not more than 2°C in a 24-h period. All feeding studies were conducted at 10°C in an environmental chamber using a 18D:6N photoperiod.

Static exposures consisting of 30 g wet sediment overlaid with 20 ml Lake Michigan water in 50 ml beakers were prepared and allowed to settle overnight before adding animals. Two fourth instar *C. riparius* larvae were added to each beaker for timed intervals of 48 h, 96 h, seven d, and ten d. Two sets of feeding exposures were created along with one set of control exposures. Two mg Cerophyl® in a water suspension were added to each Level I test beaker every third day of the assay. This dose was doubled for Level II test beakers. These feeding levels were comparable to those used in previous whole-sediment assays that employed midge larvae (Nebeker *et al.* 1984, Ankley *et al.* 1993 *a*). Control beakers received no additional food. Five replicate exposure beakers from each of the two feeding levels and control groups were sampled at the completion of each exposure interval. Individual larvae were removed from the sediment, rinsed in distilled water, blotted dry, weighed, and placed into xylene-based scintillation cocktail (3a70b; Research Products International, Inc., Mt. Prospect, IL) for direct extraction of the contaminants. Since previous elimination studies that tested fourth instar *C. riparius* larvae at 10°C showed that only a minimal amount of the contaminant was associated with gut contents, the organisms were not purged of gut contents before analysis. After standing 48 h in the cocktail, larvae were analyzed via liquid scintillation counting (LSC). Lipid content of individual animals was analyzed after two-, four-, seven-, and ten-d exposures for two of the assays (pyrene and the second DDT/chrysene assays) to determine any differences in lipid accumulation between test and control exposures, using micro-gravimetric procedures (Gardner *et al.* 1985).

Contaminant concentration and total organic carbon (TOC) in sediment from each test group and controls were also determined after each exposure interval. Sediment samples were weighed and dried at 90°C to constant weight for wet:dry weight ratios. Contaminant concentration in the sediment samples was determined by placing approximately 100 mg wet sediment directly into scintillation cocktail and sonicating the sample for two min, following the procedure of Lydy and Landrum (1993). All samples for LSC analysis were left to stand in scintillation cocktail for at least 48 h before determining activity. Samples were corrected for quench by using the external standards ratio method after subtracting background. The TOC content of sediment samples was determined by drying the sediment to constant weight, treating with 1 N HCl to remove carbonates, redrying, and assaying organic carbon on a Perkin-Elmer 2400 CHN Elemental Analyzer.

Differences in larval weight and contaminant accumulation between controls and the two feeding levels were determined for each timed interval by using Student's t tests. Differences were considered significant when $p < 0.05$.

RESULTS

Apparent steady-state concentrations of contaminants in larvae were attained prior to 240 hours in all of the assays. Steady-state concentrations in control exposures were apparently reached by 48 hours for *trans*-chlordane (Figure 1) and chrysene (Figure 2). Accumulation of pyrene in controls approached steady state by 168 hours, but decreased at 240 hours (Figure 2), although mean Feeding Level I accumulation appeared to attain steady-state by 48 hours. Larval accumulation of DDT and BaP continued to rise in controls and in both feeding levels throughout the studies (Figures 1 and 2), with the exception of control BaP exposures, where steady-state levels were attained by 168 hours. DDT accumulation was greater from sediment stored one week than from sediment stored three months (Figure 1), even though DDT concentrations in the two sediments were similar

(Table 1). However, accumulation of chrysene, the dual-labeled counterpart of DDT in the same sediments, was similar in both assays (Figure 2). Larval accumulation was proportional to contaminant concentrations found in the sediments (Figures 1 and 2, Table 1). Mean bioaccumulation ratios for controls (ng contaminant/g wet weight larvae : ng contaminant/g dry weight sediment) ranged from 0.63 (pyrene) to 2.48 (*trans*-chlordane) after 240 hours.

Contaminant accumulation in larvae differed significantly between controls and feeding levels for PAHs, but was generally not consistent among exposure intervals. Larvae in both feeding levels accumulated significantly more pyrene than controls after 48 and 240 hours ($p < 0.05$), but no significant differences were seen among feeding levels and controls at 168 hours ($p = 0.296$ and 0.217 for controls and Feeding Levels I and II, respectively; Table 2). BaP-dosed larvae showed significantly higher accumulation in both feeding levels than in controls only at 168 hours (Table 2). Conversely, larvae exposed to chrysene-dosed sediments in fed exposures accumulated less contaminant than controls after 96 (Study II), 168 (Studies I and II), and 240 hours (Studies I and II; Table 2). Accumulation was not significantly different among either of the feeding levels or controls for the two insecticides studied. Small differences in individual uptake rates and/or physiological state of the larvae may

TABLE 1. CONCENTRATION OF CONTAMINANTS IN *Chironomus riparius* LARVAE AFTER SEVEN-DAY EXPOSURES IN WHOLE SEDIMENT

Contaminant	Concentration of contaminant in sediment, ng/g dry weight	Control ng/g wet weight	Feeding Level I ng/g wet weight	Feeding Level II ng/g wet weight
Pyrene	0.640	0.6819	0.7549	0.8638
	(0.06)*	(0.298)	(0.285)	(0.320)
BaP	0.269	0.0451	0.1063**	0.0902**
	(0.01)	(0.013)	(0.040)	(0.018)
Chrysene I	32.400	35.67	28.53	18.83**
	(7.06)	(10.9)	(13.7)	(4.2)
Chrysene II	33.171	31.32	26.80	20.31**
	(2.60)	(6.6)	(7.7)	(5.9)
DDT I	381.93	428.42	553.58	364.24
	(66.43)	(140.4)	(195.8)	(125.3)
DDT II	349.82	279.51	292.91	267.96
	(53.72)	(53.89)	(103.0)	(72.0)
trans-Chlordane	1016.21	1547.53	2170.40	2008.82
	(72.22)	(968.7)	(289.0)	(506.0)

* ± 1 S. D.
** Values are significantly different from controls at $p < 0.05$.

account for the significant differences in accumulation of DDT between the two feeding levels at 168 hours in the first DDT/chrysene assay ($t = 2.494$, 16 df, $p = 0.013$; Table 2).

TABLE 2. DIFFERENCES IN CONTAMINANT ACCUMULATION BETWEEN FEEDING LEVELS I, II, AND CONTROLS, USING STUDENT'S t-TESTS FOR EACH TIMED EXPOSURE INTERVAL

Contaminant	48 Hours		96 Hours		168 Hours		240 Hours	
	A	B	A	B	A	B	A	B
Pyrene	+	+	0	+	0	0	+	+
BaP	0	0	0	0	+	+	0	0
Chrysene I	0	0	0	0	0	-	-	-
Chrysene II	0	0	-	-	0	-	0	-

A = comparison between controls and Feeding Level I, B = comparison between controls and Feeding Level II. All data were considered significant at p > 0.05. 0 = no significant difference between categories; + = accumulation significantly greater than found in controls; - = accumulation significantly lower than in controls (e. g. + in Column A indicates Feeding Level I accumulation was significantly greater than in control exposures.) No differences between feeding level I and feeding level II were found for any of the compounds studied at the p < 0.05 level of significance.

Wet weights of control larvae were not significantly different from test larvae at the completion of the assays, except for pyrene exposures. In single-labeled pyrene exposures, Feeding Level I and II larvae were an average 41.0 and 66.0% heavier than control larvae after 240 hours ($t = 2.680$, 16 df, $p = 0.0014$, control/Feeding Level I; $t = 2.812$, 16 df, $p = 0.0078$, control/Feeding Level II). Average larval wet weights in all assays rose from 9.64 ± 2.2 mg (mean $\pm$ 1 S. D.) at 48 hour to 12.84 ± 1.2 mg at the completion of the assays.

Lipid content in larvae remained similar throughout the exposure intervals and was not significantly different among either the feeding levels or controls. Larval lipids, sampled from pyrene and the second set of DDT/chrysene assays, averaged $2.99 \pm 1.3\%$, $2.93 \pm 1.3\%$, and $3.39 \pm 1.7\%$ on a dry-weight basis for Feeding Levels I, II, and controls, respectively. Dry to wet weight ratios for *C. riparius* larvae averaged 3.5.

Survival of control larvae in all assays was 100%. Survival in Feeding Level I was lowest, with 87.5% of the larvae surviving to 240 hours in the BaP/chlordane exposures, 95% surviving in pyrene and the first DDT/chrysene exposures, and 80% surviving in the second DDT/chrysene exposures. All larvae survived to 240 hours in Feeding Level II exposures except with BaP/chlordane exposures, where survival was 87.5%.

Because the amount of exogenous food added to the exposures was quite minimal, sediment total organic carbon did not change over the course of any of the assays and ranged from 0.42 to 0.53%. No trends in sediment contaminant concentrations were observed, and contaminant concentrations in sediment remained the same for all contaminants over the course of the exposures.

DISCUSSION

Feeding the test orgnisms during a bioassay was expected to reduce contaminant accumulation. When feeding is a dominant route for contaminant accumulation, changes in organism health in response to the contaminant can act to enhance or reduce ingestion and/or elimination rates. For example, stress placed on the

assay organism from transport between the culture aquaria and test exposures, or from an exposure environment unusual to the organism (*i. e.*, aqueous exposures without substrate for burrowing) may reduce its feeding rate. Conversely, feeding rate as well as overall metabolic rate may be increased as a stimulatory response to contaminant exposure. If uncontaminated food is preferentially ingested, elimination may be enhanced from differential partitioning of the contaminants to fecal material. In addition, preferential ingestion of uncontaminated food will reduce (dilute) the effective concentration of contaminants accumulated as the organism feeds to meet its
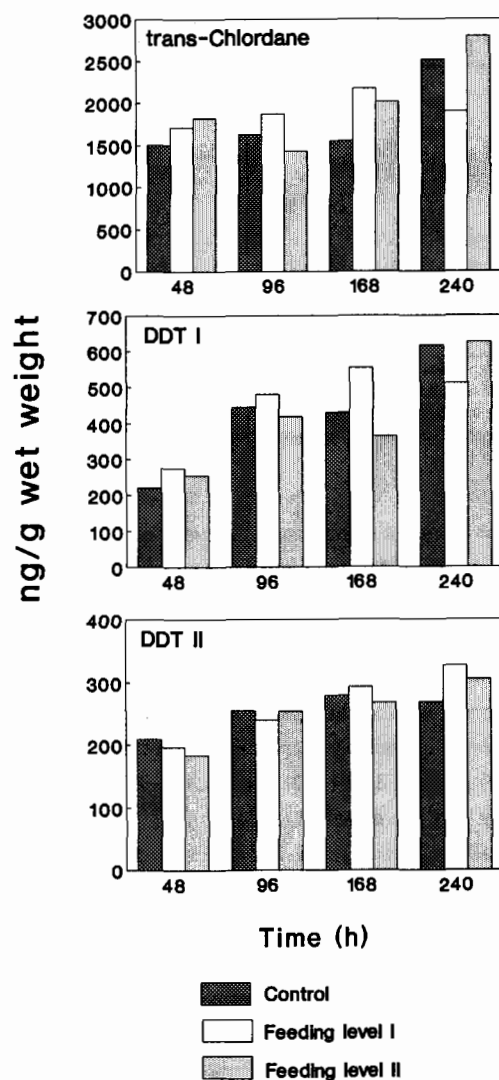
Figure 1. Mean concentration of *trans*-chlordane and DDT (two separate assays) in midge larvae after two-, four-, seven-, and ten-day exposures in whole sediment. Control = no food added, Feeding Level I = 2 mg food added per beaker, and Feeding Level II = 4 mg food added per beaker. N = ten larvae for each exposure interval.
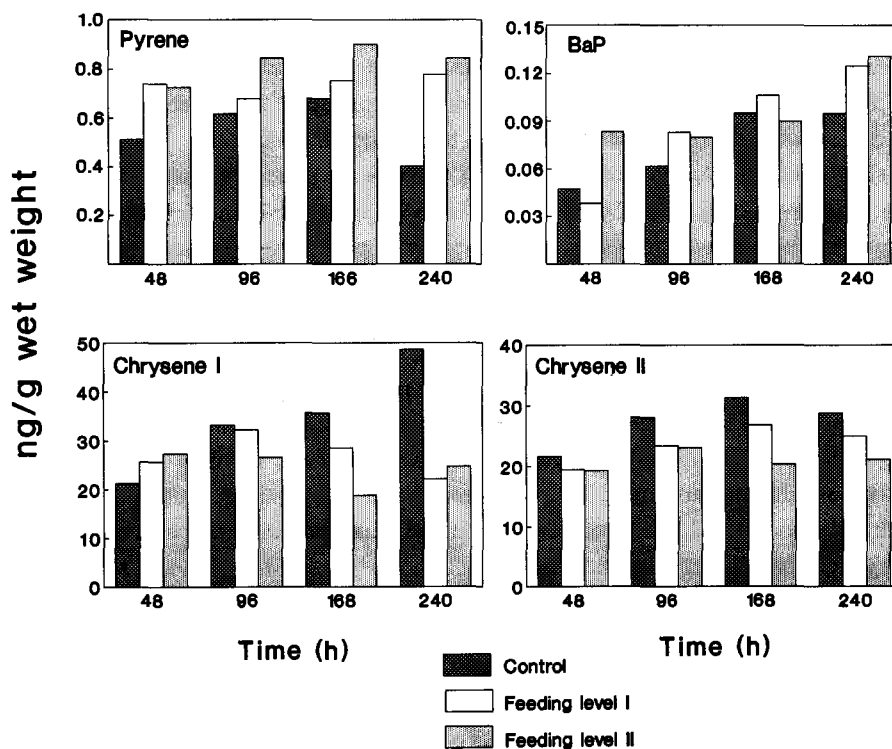
Figure 2. Mean concentration of pyrene, benzo(*a*)pyrene, and chrysene (two separate assays) in midge larvae after two-, four-, seven-, and ten-day exposures in whole sediment. Control = no food added,Feeding Level I = 2 mg food added per beaker, and Feeding Level II = 4 mg food added per beaker. N = ten larvae for each exposure interval.

nutritional requirements. Finally, the overall feeding rate in the presence of a high quality food may reduce feeding and elimination rates due to the different nutritional content of the added food compared to sediment. Thus, it is possible to see a decrease in contaminant accumulation when organisms are fed uncontaminated food during a bioassay. This may explain the results obtained in the chrysene exposures, where feeding may have resulted in a combination of decreased contaminant ingestion and increased elimination. Previous studies showed that toxicity of sediment-associated metals to oligochaetes decreased when exogenous food was added in laboratory tests (Wiederholm *et al.* 1987). This may have been a result of lower exposure or increased elimination, as observed for chrysene, or an overall improvement in organism health.

Differential accumulation in larvae between feeding levels and controls may have been due, in part, to physical and chemical characteristics of the contaminants. Contaminant bioavailability of the two insecticides, DDT and *trans*-chlordane, did not change upon addition of food to the exposures. However, differences in accumulation between feeding levels and controls were seen after at least one of the exposure periods for BaP and pyrene. The increased bioavailability of these two contaminants with feeding may be due to enhanced sorption of these PAHs onto food particles. Feeding has led to enhanced cadmium toxicity because of rapid transfer of

cadmium from test solutions onto food and food/sediment mixtures (Pascoe *et al.* 1990). Any sorption of BaP and pyrene onto uncontaminated food particles may have increased the bioavailability of these contaminants, and effectively "overridden" any reductions in contaminant ingestion resulting from decreased feeding rates, or increases in elimination due to contaminant effects (*e. g.,* higher metabolic rate).

The significant difference in accumulation of DDT between sediment aged one week and three months indicates that the bioavailability of DDT decreased with sediment aging, while that of chrysene dosed in the same sediment stayed relatively constant. Our chrysene data agree with previous studies that reported uptake clearance values for chrysene in *Diporeia* spp. not significantly different between sediments spiked three days and 150 days prior to exposures (Landrum *et al.* 1992). Other studies have reported bioavailability of PAH from sediment to significantly decline with increased contact time between the sediment and contaminants (Varanasi *et al.* 1985, Landrum 1989). The reasons for the variability among these studies are not known, and no similar studies have examined changes in DDT bioavailability with sediment aging. In this study, the concentration of DDT was an order of magnitude greater than that of chrysene in the dual-labeled sediment. These concentration differences may have affected contaminant partitioning over the three month aging period. Further studies relating the bioavailability of sediment-associated contaminants with sediment aging will need to be undertaken before an understanding of this phenomena is attained.

Larval feeding selectivity may have accounted for the variation in contaminant accumulation. Although *Chironomus* larvae are described as filter feeders, feeding on algae and detritus (Walshe 1951, Coffman 1967, Oliver 1971), very little is known about their feeding preferences. Differential sorption of the contaminants to food or sediment particles, coupled with selective ingestion of these particles, may significantly influence larval exposure, as shown in previous studies that examined the uptake of hexachlorobiphenyl and BaP in a benthic amphipod (Harkey *et al.* unpublished data).

Although the quantity of food added to the exposures in this assay was comparable to that used in some previous studies, it was much lower than amounts used in other studies. We used concentrations of 0.022 and 0.044 mg food per ml wet sediment per day for Feeding Levels I and II, respectively. Ankley *et al.* (1993 *a*) reported that optimal conditions for second instar *C. tentans* larvae in toxicity assays were obtained with the addition of 0.038 mg food per ml wet sediment per day. "Optimal" conditions were specified as those resulting in the highest survival of larvae with minimal loss of water quality. Other studies have reported adding up to seven times this amount of food to exposures (Pittinger *et al.* 1989, Ingersoll and Nelson 1990). Food concentrations at these levels have the potential to lower water quality, alter organic carbon content of the substrate, and change the bioavailability of the contaminants being tested.

An additional factor to consider in feeding studies such as these is the quality of food available to the test organisms, both that contained in the exposure sediments and that added exogenously. A major factor influencing the results of studies that examined growth, survival, and reproduction of species exposed to sediments differing in organic carbon content, particle size distribution, and chemical composition was nutrition (Wiederholm *et al.* 1987, Ankley *et al.* 1993 *a, b*, Phipps *et al.* 1993). In general, these studies demonstrated lower growth rates, reproduction, and survival in nutritionally poor substrates such as sand and sediments from oligotrophic lakes. Thus, it is suggested that food be added to nutritionally poor sediments during bioassays in order to avoid results that confound nutritional quality with contaminant toxicity (Phipps *et al.* 1993). However, the low organic carbon content of the sediment in this study (approx. 0.47%) appears to have had little effect on growth and lipid content of the organisms.

Published guidelines for conducting toxicity assays with *Chironomus* spp. recommend that first to second instar larvae be used (ASTM 1991 *a*). Fourth instar larvae were used in this study due to the ease of identifying and handling the organisms. However, individual *Chironomus* sp. in fourth larval instars may be at very different

stages of development (Wülker and Götz 1968). The variation in contaminant accumulation, especially that noted between 168-and 240-hour exposure periods, may be attributed to changes in physiology and metabolic rates among the groups of individuals used in this study. In addition, significant differences in accumulation of contaminants between fed and unfed animals in bioassays will change with the species and life stage used, as well as with the temperature at which the assay is conducted. Most midge studies have been run at room temperature; therefore, the results of this study cannot accurately be extrapolated to previous assays conducted at 22 - 25°C. Any statistically significant differences in contaminant accumulation may be more or less marked under different environmental conditions. Consequently, the relative magnitude of such statistical differences becomes a point of concern with the development of standard bioassay procedures. Further research will be needed to describe the environmental conditions (e. g., temperature, organism density, sediment volume) that most favorably define such endpoints as accumulation, growth rate, and weight gain.

Bioaccumulation models of hydrophobic organic contaminants have made the assumption that compounds will partition to organic matter in proportion to their respective hydrophobicities, as measured by log K_{ow}. However, most bioaccumulation models assume that partitioning of contaminants is at equilibrium during the time of exposure. If equilibrium is attained rapidly (e. g., partitioning to added food particles happens quickly), then the behavior of hydrophobic non-polar compounds should behave consistently in proportion to the hydrophobic nature of the contaminant. The preliminary data from this study did not exhibit consistancy. Rather, the compounds behaved differently both between compound classes (chlorinated hydrocarbons and PAHs) and within a compound class (PAHs). The inconsistancies among compounds do not exhibit any particular pattern with respect to the hydrophobicity of the compound, so hydrophobicity alone is insufficient to explain the variance either within the PAH group or between compound classes. The absence of simple partitioning relationships to explain the differential accumulation of hydrophobic contaminants leaves us speculating about the mechanisms and processes that led to these data. Potential explanations include differential partitioning among particle types, selective ingestion, and alteration of accumulation and elimination kinetics. Certainly, further research on the route of exposure and the partitioning among particles will aid in understanding the exposure potential for various compounds. Until the factors that influence the variance in exposure are identified and can be accounted for, the influence of feeding in toxicity bioassays will make the exposure, and therefore the results, poorly interpretable.

ACKNOWLEDGEMENTS

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